Original Report

Phenotypic diversity of enterotoxigenic *Escherichia coli* (ETEC) isolated from cases of travelers' diarrhea in Kenya

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Background: The aim of this study was to characterize phenotypically enterotoxins, colonization factors (CFs) and the antibiotic susceptibility of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from cases of acute diarrhea that occurred in Europeans traveling to resorts in Mombasa, Kenya; this information is critical for the development of vaccines and empirical treatment.

Methods: Over a 1-year period from 1996 to 1997, five *E. coli*-like colonies were obtained from each of 463 cases with acute diarrhea. These strains were characterized for enterotoxins using GM-1 ELISA, for CFs using a dot-blot assay, and for antibiotic susceptibility using antibiotic disks.

Results: Of 164 strains characterized for ETEC phenotype, 30 (18%) expressed heat-labile toxin (LT) only, 83 (51%) heat-stable toxin (ST) only, and 51 (31%) both LT and ST. Analysis for CF expression demonstrated that 107 (65%) of the strains were positive for CFs, including CFA/IV (46%), CFA/II (35%), and CFA/I (5%), while less than 4% expressed less common CFs. All ETEC strains tested were resistant to erythromycin and sensitive to ceftriaxone. Over one-third of the strains were resistant to sulfamethoxazole–trimethoprim or tetracycline. Six strains were resistant to nalidixic acid; none of these were resistant to ciprofloxacin.

Conclusions: Cumulatively, our findings indicate that ETEC in this region comprises a highly diverse group of bacterial enteropathogens, and that the development of prophylactic agents against ETEC faces major challenges because of this diversity.

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INTRODUCTION

Despite its discovery and initial characterization nearly 30 years ago,[1] enterotoxigenic *Escherichia coli* (ETEC) remains the most common cause of acute diarrhea among children in developing countries and travelers to such areas.[2–19] Annually, an estimated 400 million episodes of diarrhea and up to 700 000 deaths are attributed to ETEC infections.[7] The virulent nature of the infection is a function of two factors, enterotoxins and specific adhesins called colonization factors (CFs), which work in concert to induce diarrhea. Both types of virulence factor are generally plasmid encoded.[20,21]

CFs enable ETEC to adhere to specific receptors located on the surface of epithelial cells of the small intestine, permitting colonization.[22–24] CFs commonly expressed by ETEC strains isolated from human disease include CFA/I, CFA/II, CFA/IV, CS12 (PCFO159), CS14 (PCFO166), and CS17. Several other CFs have been identified, including CS7, CS8 (CFA/III), CS10 (2230), CS11 (PCFO148), CS13 (PCFO9), CS18 (PCFO20), CS19, CS20, and CS21 (Longus). CFA/II strains can express CS1+CS3 and CS2+CS3 or CS3 alone, and CFA/IV strains can express CS4+CS6 or CS5+CS6 or CS6 only.

ETEC strains also express one or both of two enterotoxins: a heat-stable toxin (ST), and a heat-labile toxin (LT), which act at the epithelial surface of the small intestine.[25,26] LT is an immunogenic protein that is structurally, functionally and antigenically related to cholera toxin.[27] ST is a non-immunogenic protein of 18–20 amino acids that is functionally and structurally related to the mammalian protein guanylin.[28] Expression of LT or ST initiates a sequence of events resulting in the activation of adenylate cyclase or guanylate cyclase

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and increased levels of cAMP or cGMP, respectively.[25,26] As a result, disturbances in electrolyte balance take place, with the resulting fluid secretion causing watery diarrhea.

While the diarrhea caused by ETEC is usually of short duration and self-limiting, it is a significant cause of morbidity and incapacity among Western travelers to endemic regions, and of mortality among young children resident in these regions. While antibiotics are often used to alleviate the symptoms of acute infection, limited and often outdated information, compounded by increasing drug resistance, has made empirical treatment difficult. Several prophylactic and treatment drug regimens have been described for ETEC diarrheal disease,[29,30] with quinolones being the current drugs of choice for both prophylaxis and treatment. However, the use of quinolones in the pediatric population remains controversial. Therefore, other methods, including vaccine development, have been aggressively pursued for the control of ETEC infections. To develop a vaccine offering the broadest protective potential, and to assess the extent of antibiotic resistance, the characterization of representative ETEC strains from different geographic regions is a necessity.

Previous epidemiologic surveys conducted in community-based settings within distinct geographic areas of South America, Asia and the Middle East endemic for ETEC have shown substantial variation in the distribution of ETEC toxin types and CFs. Expression of ST and LT varied from 24% to 65%, and from 21% to 50%, respectively, while the expression of LTST varied from 9% to 27%.[5,17,31] These studies also reported that up to 50% of ETEC strains apparently did not express any CFs, suggesting a potential protective gap in current vaccine prototypes. However, little information is available on toxin and CF phenotype or antibiotic resistance of ETEC strains isolated from travelers to Africa, another region endemic for ETEC.[19,32] Therefore, in the present study, we report the characterization of ETEC strains obtained from cases of diarrhea that occurred in European tourists visiting resorts in Mombasa, Kenya.

MATERIALS AND METHODS

Bacterial strains

As part of a study to determine the prevalence of diarrhea in travelers, Europeans vacationing in Mombasa, Kenya between September 1996 and August 1997 were asked to come to a local clinic for evaluation should they develop diarrhea during their stay.[33] After the patient was evaluated in the clinic, they were asked to provide a stool specimen for analysis. A sample of stool was streaked onto bacterial culture media, including MacConkey's and blood agar plates. From these plates, five lactose-positive *E. coli*-like colonies were selected and individually stored at −70°C in brain heart infusion broth supplemented with 15% glycerol. At regular intervals, these lactose-positive isolates were shipped to the Naval Medical Research Unit (3), Cairo, Egypt for ETEC testing, using a GM1 ELISA assay to detect either ST or LT, as previously described.[34,35]

For each set of five isolates tested, if any were positive for an enterotoxin, the patient was said to have had ETEC-associated diarrhea, and the toxin-positive isolates were tested for CF expression, using monoclonal antibodies against CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS14, and CS17.[36–38] The following reference strains were used as controls for enterotoxin and CFA expression: 286C2 (LT), ST64111 (ST), 258909-3 (LTST and CFA/I), E1392 (CS1), E278485/2 (CS2), VM73494 (CS3), E11881/9 (CS4), E17018A (CS5), VM75688 (CS6), E29101A (CS7), E34420A (CS8), E350C1A (CS12), E3476A (CS14), E20738A (CS17), and HS4, a commensal strain isolated from a human volunteer. If all the isolates from a given case of diarrhea had the same toxin and CF phenotype, then one isolate was selected as representative. In two diarrhea cases, ETEC isolates were found to be expressing different combinations of toxins. From these cases of mixed ETEC infection, two isolates representative of the different toxin and CF profiles identified were selected. In total, 164 ETEC strains from 162 diarrheal cases were characterized.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the ETEC isolates for ceftriaxone, gentamicin, erythromycin, tetracycline, nalidixic acid, ciprofloxacin and trimethoprim–sulfamethoxazole was performed, using the disk diffusion method.[39] Those strains found to be resistant to nalidixic acid were also tested for susceptibility to ciprofloxacin. NCCLS guidelines were used to determine whether an isolate was sensitive, intermediate–sensitive, or resistant.[40] For analysis, isolates with an intermediate zone of resistance to an antibiotic were considered to be resistant to that drug.

Statistical analysis

Data analyses were performed using Pearson's chi-square test programmed into the spreadsheet module of AppleWorks (Apple Computer, Cupertino, California, USA).

RESULTS

Over a 1-year period, 463 cases of diarrhea were reported from which a stool specimen was processed for pathogens. ETEC was identified as the pathogen in 162 of these cases. From the 162 cases of ETEC-mediated diarrhea, 164 isolates were selected as representative strains. Eighteen per cent (30/164) of the strains expressed LT only, 51% (83/164) ST only, and 31% (51/164) both LT and ST. Sixty-five per cent

(107/164) of these strains expressed a known CF (Figure 1). The remaining 35% (57/164) of the strains were phenotypically negative for a CF. Among the CF-positive isolates, 46% (49/107) were CFA/IV positive, 35% (37/107) CFA/II positive, and 5% (5/107) CFA/I positive, while less than 4% expressed CS7 (3/107), CS8 (4/107), CS12 (2/107), CS14 (4/107), or CS17 (3/107). Of the CFA/IV-positive isolates, 90% (44/49) expressed CS6 only, 6% (3/49) CS4+CS6, and 4% (2/49) CS5+CS6. Of the CFA/II-positive isolates, 5% (2/37) expressed CS3 only, 70% (26/37) CS1+CS3, and 24% (9/37) CS2+CS3. All four ETEC strains that were positive for CS8 also co-expressed CS6.

A higher percentage of the ST-only and LT+ST ETEC strains expressed known CFs (70%, 58/83, and 71%, 36/51, respectively) as compared to LT-only strains (43%, 13/30). Among 91 strains that expressed CFA/I, CFA/II, or CFA/IV, 87 of 91 (96%) strains expressed ST either as the sole toxin or in combination with LT (Table 1), whereas 4 of 91 (4%) expressed LT only, and CS6 was the only antigen detected. Fifty-seven of the 164 (35%) ETEC strains did not express any detectable CFs: 17 expressed LT only, 25 expressed ST only, and 15 expressed both LT and ST.

One hundred and fifty-seven of the 164 (96%) ETEC strains were tested for antibiotic resistance. No statistically significant correlation between antibiotic resistance pattern and enterotoxin or CF expressed could be discerned (all $P>0.1$). All strains were resistant to erythromycin and sensitive to ceftriaxone or ciprofloxacin. Sixty-two strains (39%) were resistant to trimethoprim–sulfamethoxazole, and 66 (42%) were resistant to tetracycline, while 38 (24%) strains were resistant to both of these antibiotics in addition to

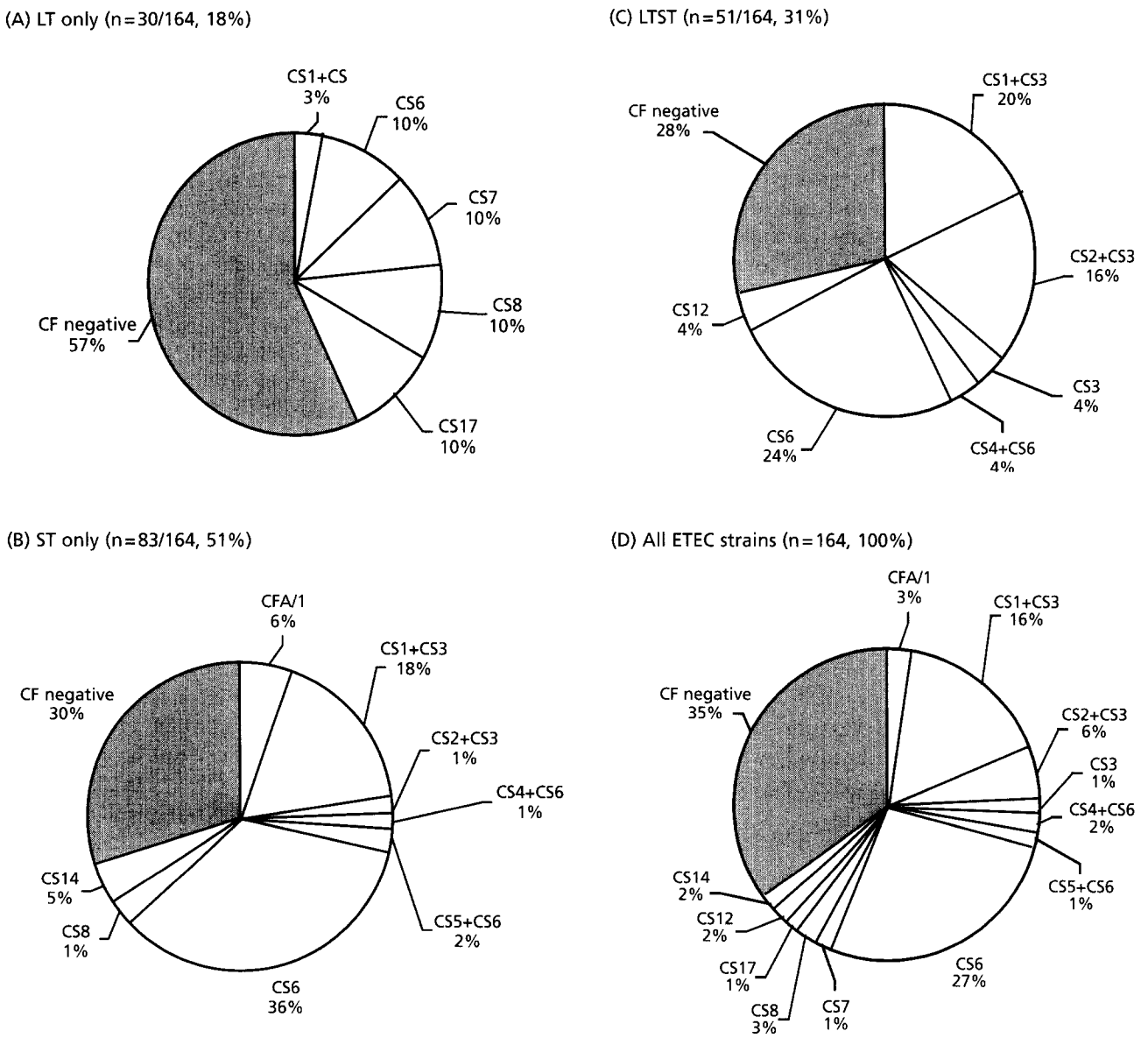

Figure 1. Distribution of the expressed coli surface (CS) antigens in relation to toxin phenotype: LT (A), ST (B), and LT+ST (C). (D) demonstrates the distribution of the expressed CS for all ETEC strains isolated from travelers suffering from aqueous diarrhea.

Table 1. Phenotypic profile of ETEC strains in relation to rCTB- and CF-based vaccine components.

Colonization factors	*LT (%)*	*LTST (%)*	*ST (%)*	*Total (%)*
CFA/I and CS1–CS6	4 (2.4)	34 (20.7)	53 (32.3)	91 (55.5)
CS17, CS8 and CS17	9 (5.5)	–	1 (0.6)	10 (6.1)
CS12 and CS14	–	2 (1.2)	4 (2.4)	6 (3.7)
Negative CFs	17 (10.4)	15 (9.1)	25 (15.2)	57 (34.8)
Total (%)	30 (18.3)	51 (31.1)	83 (50.6)	164 (100.0)

erythromycin. A single strain (<1%) was resistant to gentamicin, while six strains (4%) were resistant to nalidixic acid. All of the nalidixic acid-resistant strains were susceptible to ciprofloxacin. Five strains (3%) were resistant to four antibiotics. Four of these were resistant to erythromycin, nalidixic acid, trimethoprim–sulfamethoxazole, and tetracycline. The other strain was resistant to erythromycin, gentamicin, nalidixic acid, and tetracycline.

DISCUSSION

Past phenotypic characterization studies of ETEC isolated from Africa have focused on the analysis of enterotoxin, but not CF, expression.[2,9,10,17] This limitation makes it difficult to assess the protective potential of existing vaccine prototypes and to decide which components to add to future vaccines. Furthermore, data in the literature are limited with regard to the antibiotic susceptibilities of ETEC pathogens. In this study, we have phenotypically characterized ETEC strains from defined cases of acute diarrhea in Western travelers over a 1-year period with respect to enterotoxin production, CF expression, and antibiotic susceptibility.

Previous studies have indicated that many antibiotics commonly prescribed for acute diarrhea are ineffective, due to the high prevalence of multiresistant *E. coli*.[11,16,41] We have demonstrated that ETEC strains from Egypt are routinely resistant to ampicillin, streptomycin, and chloramphenicol (David et al, unpublished data). Some investigators have reported an association between multiresistance and enterotoxin phenotype. DeBoy et al found that multiresistance occurred more often in ST-producing strains,[32] while Turner et al observed that such resistance was more common in LT-producing strains.[42] In the current study, we were unable to discern any relationship between drug resistance and the type of toxin produced.

The pattern of enterotoxin expression worldwide is such that ST-only ETEC strains are more common than either LT-only or LTST strains.[43] The phenotypic distribution of our ETEC strains is in agreement with this global trend. However, regional variations in ETEC enterotoxin phenotype abound, even within restricted geographic areas.[2,8,44] While a study in rural Egypt found that LT-only strains comprised 60% of all ETEC strains, and ST-only strains comprised 36%, a subsequent study found ST-only strains to outnumber LT-only strains by nearly two to one.[2,17] A similar paradox was seen in studies conducted on deployed Western forces during the Gulf War. Among US troops, nearly half of the ETEC strains isolated expressed both LT and ST, and of the balance, 39% expressed ST only, and 17% expressed LT only.[9] In contrast, 73% of the ETEC strains isolated from neighboring UK troops expressed ST only.[11]

Similarly, surveys of ETEC strains from different regions have shown a wide variation in the percentage of strains expressing CFs. The current study, with 65% of the strains expressing a known CF, was at the higher end of reports from the literature, which give values ranging from approximately 20% to 75%.[3,5,12,17,19,44,45] The predominant CF that we identified was CFA/IV, with CS6 alone as the principal antigen detected, followed by CFA/II, with CS3+CS1 the most common combination. ETEC strains expressing CFA/I or other CF antigens were found in only a few cases in this study. This distribution is similar to our previous findings from a community-based study in Northern Africa,[17] but differs from that seen during the Gulf War, in which Wolf et al[9] identified a higher percentage of ETEC strains that expressed CFA/II, compared to those that expressed CFA/IV. While some studies have found antigens such as CS8, CS12, CS14 or CS17 comprising a substantial percentage of the CFs detected, we found that they constituted only a small proportion of the total CFs.[23,24,43] Interestingly, we found that CS8 was always co-expressed with CS6, a rarely reported combination of antigens.

Previous studies have found that CF expression is more likely to be found among ST-producing strains,[23,24,43,44,46] while LT-positive strains are more likely to be CF negative, as a result of either the loss of a plasmid encoding for a CF, or the expression of a less common or, as yet unidentified, CF.[12,47] In this study, a high percentage (53%) of the ST-associated ETEC strains expressed the most common CFs, namely CFA/I and CS1-6. Common epitopes between members of the CFA/I family, namely CS1, CS2, CS4, CS14, CS17, and CS19, have been demonstrated.[24,48] Such cross-reactions may induce an antibody response, not only to homologous but also to heterologous CFs.[49,50] Furthermore, a protective immunity to ETEC strains (LT positive), in particular strains expressing CS7 and CS17, has been demonstrated to be age associated in primed children.[19,51] Ten per cent of the isolated ETEC strains

were LT or ST associated, and expressed less common CFs. The remaining 35% of the ETEC strains (10% LT only and 25% ST associated) did not express known CFs, and most of these expressed LT, corroborating the earlier studies. We have examined some of the apparently CF-negative ETEC strains, and found that many appear to express previously undescribed CFs[47] (Khalil et al, unpublished data).

In the context of the development and testing of candidate anti-ETEC vaccines, our findings indicate that the formulation of a broadly protective vaccine against ETEC poses major challenges because of the strain diversity. As anti-LT and CF immunity both play a role in protection against ETEC disease,[52,53] the current prototype ETEC vaccine is designed to reflect this observation. The toxoid component of the prototype ETEC vaccine, recombinant cholera toxin 'B' subunit (rCTB), induces a considerable anti-LT immunity that encompasses LT-positive strains, but leaves ST-only strains untargeted. To circumvent this protective gap, CFA/I and CS1-6 are included in the vaccine formulation.[54–56] Following this line of reasoning, our findings suggest that such an ETEC vaccine will be at least 75% protective against regional ETEC isolates (Table 1). The remaining 25% of the ETEC strains are ST associated and CF negative. These strains need further characterization with the use of antibody probes for the detection of the less common CFs, including CS10, CS11, CS13, CS18, CS19, CS20, and CS21, to determine the incidence of such CFs in regional endemic areas for vaccine purposes. Thus, phenotypic characterization of representative ETEC strains from diverse geographic regions endemic for ETEC, particularly for CFs, remains a critical prerequisite for the assessment of the protective potential of current prototypes and for the design of future vaccines. For this reason alone, the identification and characterization of new CFs is critical to enhance the potential protective scope of prototype anti-ETEC vaccines and to develop tools needed to determine the relative prevalence of different CFs on a regional basis.

Our findings show that, phenotypically, ETEC is a complex and diverse pathogen, corroborating results reported elsewhere.[12,14,17,57] This apparent complexity poses major challenges for prophylactic and preventive measures. Among the diversity, we were still able to detect combinations of enterotoxin and CF that occurred more frequently, most notably LT-only strains with CS8+CS6, CS7, or CS17, LTST strains with CFA/II, CFA/IV, or CS14, and ST-only strains with CFA/I, CFA/II, CFA/IV, or CS12. The potential adaptive advantages, if any, of these preferred combinations are unknown. Nevertheless, the phenotypic diversity of ETEC makes it clear that an effective and broadly protective vaccine must incorporate multiple protective antigens and perhaps also be designed to take into account specific regional phenotypic variations.

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This study was approved by institutional review boards at the Naval Medical Research Unit No. 3 in compliance with all Federal regulations governing the protection of human subjects. All subjects or their guardians gave voluntary, informed consent for participation prior to enrollment in this study. The KEMRI's Ethical Review Committee and the University of Zurich Ethical Review Committee also approved this study.

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